

## Negative spatial regulation of the lineage specific *CyIIIa* actin gene in the sea urchin embryo

BARBARA R. HOUGH-EVANS, ROBERTA R. FRANKS, ROBERT W. ZELLER, ROY J. BRITTEN and ERIC H. DAVIDSON

*Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA*

### Summary

The *CyIIIa*·CAT fusion gene was injected into *Strongylocentrotus purpuratus* eggs, together with excess ligated competitor sequences representing subregions of the *CyIIIa* regulatory domain. In this construct, the chloramphenicol acetyltransferase (CAT) reporter gene is placed under the control of the 2300 nucleotide upstream regulatory domain of the lineage-specific *CyIIIa* cytoskeletal actin gene. CAT mRNA was detected by *in situ* hybridization in serial sections of pluteus stage embryos derived from the injected eggs. When carrier DNA lacking competitor *CyIIIa* fragments was coinjected with *CyIIIa*·CAT, CAT mRNA was observed exclusively in aboral ectoderm cells, i.e. the territory in which the *CyIIIa* gene itself is normally expressed (as also reported by us previously). The same result was

obtained when five of seven different competitor subfragments bearing sites of DNA–protein interaction were coinjected. However, coinjection of excess quantities of either of two widely separated, nonhomologous fragments of the *CyIIIa* regulatory domain produced a dramatic ectopic expression of CAT mRNA in the recipient embryos. CAT mRNA was observed in gut, mesenchyme cells and oral ectoderm in these embryos. We conclude that these fragments contain regulatory sites that negatively control spatial expression of the *CyIIIa* gene.

Key words: sea urchin embryo, lineage specific, *CyIIIa* actin gene, negative spatial regulation.

### Introduction

The *CyIIIa* cytoskeletal actin gene of the sea urchin *Strongylocentrotus purpuratus* is expressed during early embryogenesis only in the eleven cell lineages that constitute the aboral ectoderm of the pluteus (Cox *et al.* 1986; Cameron *et al.* 1989a; reviewed by Davidson, 1989). Control of *CyIIIa* transcription depends on the interaction with endogenous factors of *cis*-regulatory sequences located within a 2300 nucleotide 5' regulatory domain, as shown by gene transfer experiments carried out with a *CyIIIa*·CAT fusion gene construct (Flytzanis *et al.* 1987; Hough-Evans *et al.* 1987). Detailed mapping studies have revealed about 20 sites within this domain where nuclear proteins bind with high specificity *in vitro* (Calzone *et al.* 1988; Thézé *et al.* 1990). These fall into 13 distinct sequence classes, suggesting that this number of different factors is required *in vivo*. As part of an effort to determine the biological significance of the individual elements of this complex regulatory system, we have determined the effect on *CyIIIa*·CAT expression *in vivo* of competition for individual factors or sets of factors. Individual subfragments of the regulatory domain, each containing one or a few factor binding sites, were ligated together

with carrier DNA, and coinjected in excess with respect to the *CyIIIa*·CAT fusion into unfertilized eggs. In the accompanying paper, Franks *et al.* (1990) show that individual subfragments including the specific binding sites for the factors P7I, P5 or P4, each competitively depress *CyIIIa*·CAT expression, as does a subfragment that includes sites for two different factors called P2I and P2II, and a subfragment that includes three other sites, for factors P8I, P8II and P8III. Interactions with these factors (or a least one of the P2 factors and at least one of the P8 factors) evidently are *required* for normal levels of *CyIIIa*·CAT expression. Thus these interactions mediate positive regulatory functions. Of the remaining factors, interaction with P3B is necessary for embryonic viability (Franks *et al.* 1990), while no significant competitive depression of the level of CAT expression was observed for subfragments bearing the P7II, P3A, P1 or P6 binding sites.

In this paper, we describe the competitive effects of the *CyIIIa* regulatory subfragments on the *spatial* rather than the quantitative expression of *CyIIIa*·CAT. The cell types in which CAT mRNA is expressed in embryos bearing excess competitor fragments were determined by *in situ* hybridization, carried out on serial sections made at an advanced stage of embryonic development.

No qualitative effect on spatial expression was observed with any of the subfragments found by Franks *et al.* (1990) to compete for positively acting factors, *viz.* those listed above. However, coinjection of two wholly nonhomologous competitor subfragments, one containing binding sites for the factors P3B and P3A, and the other binding sites for factor P7II, resulted in a remarkable derangement of the spatial pattern of CyIIIa · CAT expression.

## Materials and methods

### Eggs and embryos

Gametes were collected and eggs injected as described previously (McMahon *et al.* 1985; Franks *et al.* 1990). The eggs were microinjected with approximately 2 pl of DNA solution, either before or after fertilization, in place in the Petri dish. Embryo samples were taken after hatching (which occurs at 18 h postfertilization) for CAT assays, and at 72 h for *in situ* hybridization.

### Microinjection solutions

Solutions for injection contained 40 % glycerol, 250–1500 molecules of a CyIIIa · CAT fusion gene per pl, and competitor and/or sea urchin carrier DNA, exactly as described in detail in the accompanying paper (Franks *et al.* 1990).

### CAT assays

CAT enzyme content per embryo was estimated on samples of about 50 hatched blastulae, by a method derived from that of Gorman *et al.* (1982), as described by McMahon *et al.* (1984) and by Franks *et al.* (1990).

### Cytological preparation

Preparation of embryo sections and microscope slides were as described previously (Hough-Evans *et al.* 1987, based on the method of Cox *et al.* 1984). Embryos that developed from eggs that had been microinjected with CyIIIa · CAT or with CyIIIa · CAT plus competitor subfragments were fixed at the desired stage (in most cases 72 h pluteus) in 1 % glutaraldehyde (or in 2 % paraformaldehyde, 0.5 % glutaraldehyde) in buffer containing 2.5 % NaCl, 25 mM phosphate buffer pH 7.5. Embryos enclosed in agarose boxes were embedded in Paraplast (Monojet) or Tissueprep 2 (Fisher Scientific) and sectioned at 5 µm, and serial sections were placed on polysine-coated slides.

### In situ hybridization

The single-stranded antisense CAT RNA hybridization probes used here were transcribed as described previously (Hough-Evans *et al.* 1987) from a fragment of CAT coding sequence inserted into the polylinker of the pSP65 vector. Either <sup>3</sup>H- or <sup>35</sup>S-labeled nucleotides were incorporated, as indicated. *In situ* hybridization with tritiated probes was carried out as in earlier experiments by the procedures of Angerer and Angerer (1981) and Cox *et al.* (1984). These procedures were modified for <sup>35</sup>S-labeled probes by the addition of 5 mM dithiothreitol and 1 % β-mercaptoethanol to the hybridization solution, hybridization in an N<sub>2</sub> atmosphere, and posthybridization washes that included 1 % β-mercaptoethanol. The photographic emulsion (Kodak NTB-2) was diluted with three parts distilled water in <sup>3</sup>H-probe experiments, and a 1:1 dilution was used in experiments carried out with <sup>35</sup>S-labeled probe.

## Results

Our object in these experiments was to identify regions of the CyIIIa regulatory domain that might be required in normal embryos to *prevent* expression of the CyIIIa gene (i.e. of CyIIIa · CAT), in any but the aboral ectoderm cell lineages. The consequence of effective competition for a regulatory factor that mediates negative control of spatial CyIIIa expression would be to induce ectopic expression in other lineages. Effective competition of course requires that the competitor sequences be present in sufficient quantity within the relevant nuclei to titrate out a biologically significant fraction of the respective factor molecules. Franks *et al.* (1990) showed that this condition can indeed be met for many different positively acting factors. In the experiments described here, embryos taken from the same competitor injection series as studied quantitatively for CAT enzyme expression by Franks *et al.* (1990) were allowed to develop to the 70–72 h pluteus stage, at which stage serially sectioned material provides an unequivocal orientation and identification of anatomical features and tissues.

An extensive series of control experiments confirmed that for the protocols, materials and procedures utilized in this work, CyIIIa · CAT expression is normally confined to cells of the aboral ectoderm. A quantitative summary of the results of all the available CyIIIa · CAT controls is given in Table 1. Using the criterion that label must be present in cells of *two or more adjacent sections* (to avoid artifacts of occasional random sticking of labeled particulates), we found that in this recent set of experiments (Table 1, expts 4–6), 81 % of all serially sectioned embryos display cells containing significant detectable CAT mRNA. *All* of these displayed CAT mRNA in aboral ectoderm, furthermore. Three embryos, representing 2.5 % of those studied in the

**Table 1.** Expression of CyIIIa · CAT in pluteus-stage embryos developed from injected eggs

Experiment no.	Isotope	Labeled/Scored*	Labeled in aboral ectoderm	Ectopically labeled†
1	<sup>3</sup> H	6/26	6	0
2	<sup>3</sup> H	28/50	28	0
3	<sup>3</sup> H	7/7	7	0
4	<sup>3</sup> H	9/9	9	0
5	<sup>35</sup> S	93/118	93	1
6	<sup>3</sup> H	16/19	16	2
Total:		159/229		3

Embryos labeled in aboral ectoderm/Σ labeled=159/159=100 %.

Embryos labeled ectopically/Σ labeled=3/159=2 %.

#### Notes:

\* Only embryos represented by 9 or more sections were scored. Embryos were scored as labeled only if *labeled cells appeared in two or more adjacent sections*. In later experiments (experiments 1 and 2 are from Hough-Evans *et al.* 1987) a larger proportion of injected embryos were labeled, probably due to improved injection and hybridization procedures.

† Embryos were scored as being ectopically labeled if labeled cells were seen in tissue other than aboral ectoderm, in more than one section.

recent experiments (Table 1), also displayed CAT mRNA in a few cells of other types.

Examples of control embryo sections, in which significant fractions of the visible aboral ectoderm can be seen to contain CAT mRNA, are shown in Fig. 1. Gut, mesenchyme and oral ectoderm are unlabeled in these *in situ* hybridizations. The single injection gene transfer methodology used in these experiments results in mosaic DNA incorporation patterns. Hough-Evans *et al.* (1988) deduced from direct observations made by DNA *in situ* hybridization to the exogenous sequences, and other prior evidence, that most probably, in about three-fourths of recipient embryos, a single concatenate of the injected DNA is stably incorporated in a single blastomere nucleus after 1st, 2nd, 3rd or 4th cleavage. However, exogenous DNA incorporation is random with respect to cell type and cell lineage (Sucov *et al.* 1988; Franks *et al.* 1988a,b; Livant *et al.* 1988). In some cases, the labeling patterns shown in Fig. 1 of this paper permit us to identify those blastomeres of the early cleavage embryo that in fact incorporated the exogenous DNA, given knowledge of the regional cell lineage of the aboral ectoderm (Cameron *et al.* 1987; 1989a,b). For example, perusal of other serial sections (not shown) of the embryo illustrated in Fig. 1a, upper right, indicate that the incorporation appears to have occurred in one of the two first cleavage blastomeres, *viz.*, that giving rise to the 3rd cleavage blastomeres VA, VL<sub>R</sub> and the overlying Na and NL<sub>R</sub> blastomeres, while in the Fig. 1a lower embryo, incorporation appears to have been confined to the single 3rd cleavage VA blastomere. This evidence is important in a specific way for our present concerns, since the VA progeny that give rise to aboral ectoderm do not segregate from those giving rise to gut until 6th cleavage, and from those giving rise to skeletogenic mesenchyme until 4th cleavage (Cameron *et al.* 1987). Since all aboral ectoderm regions of the VA domain express CAT mRNA in this embryo, the exogenous DNA must also have been present in gut (and probably mesenchyme cells). In these locations, however, the CyIIIa·CAT gene is manifestly silent, an excellent demonstration of differential expression. Similar analyses of the lineage affiliations of labeled cells in other embryos shown are given in the legend to Fig. 1. These observations leave no doubt that, as reported earlier, CyIIIa·CAT expression is repressed in gut and mesenchyme, in the same embryos in which it is active in aboral ectoderm. Furthermore, embryos in which large NL aboral ectoderm domains are labeled, but the adjacent ciliary band regions of the oral ectoderm do not express CAT mRNA (e.g. Fig. 1e) show that CyIIIa·CAT is not expressed in the progeny of the 5th cleavage NL1u and NL2u blastomeres, while it is expressed in the progeny of their sister cells, NL1l and NL2l (Cameron *et al.* 1989a). However, this form of evidence cannot be applied to the lack of expression of CyIIIa·CAT in the main facial and stomodeal region of the oral ectoderm, as illustrated clearly in the embryos shown in Fig. 1a–b (lower), 1f, 1i, and 1j. These regions descend from the single 3rd cleavage blastomere No, which gives rise only

to facial and stomadeal oral ectoderm progeny. Thus, were incorporation of any of the exogenous CyIIIa·CAT DNA to occur in the No blastomere (or its progeny), the resulting pluteus would be scored in these experiments as unlabeled. For all incorporations in the No domain at or after 3rd cleavage (i.e. the majority; Hough-Evans *et al.* 1988) the expectation would thus be that 12.5 % of the successfully injected embryos would display no CAT mRNA, and perhaps this factor largely accounts for the observation that in Table 1 (expts 3–6) 19 % of the embryos were scored as silent with respect to CAT mRNA expression.

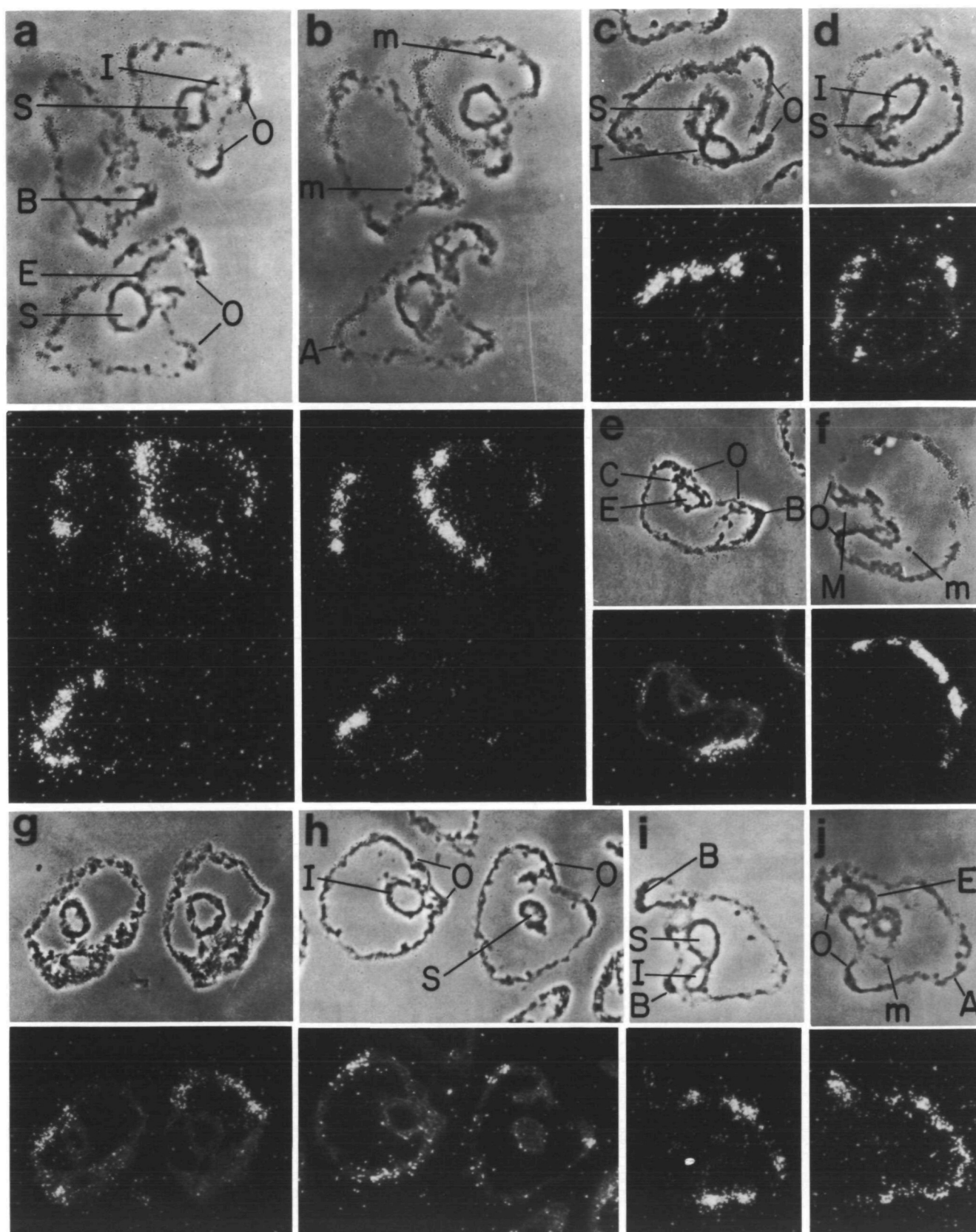
#### *In vivo competitions that do not affect spatial expression*

Spatial expression of CyIIIa·CAT was assessed in those samples of embryos that contained the maximum amounts of competitor DNA in the quantitative competition studies of Franks *et al.* (1990). Table 2 shows that in most cases, whether or not CAT expression was depressed by the introduction of competitor fragments, the spatial pattern of CyIIIa·CAT expression was not affected. That is, CAT mRNA was confined to aboral ectoderm just as in the control series summarized in Fig. 1 and Table 1. We conclude that there is no evidence from these experiments that the DNA–protein interactions mediated by the subfragments, which include the sites for factors P8I, II and III; P4; P5; P2I or P2II, affect spatial expression of CyIIIa·CAT *negatively*, because no significant ectopic expression occurred. In each of these cases, we know that the *in vivo* competition was effective (Franks *et al.* 1990). Nor was spatial expression of CyIIIa·CAT affected in the P6 competition sample; however, ‘competition’ with this subfragment also failed to depress the level of CyIIIa·CAT expression in the experiments of Franks *et al.* (1990). Therefore, for this case we lack independent evidence that the competition was effective. A null control subfragment (designated ‘0’; Thézé *et al.* 1990) which contains no known sites of DNA–protein interaction, also failed to alter spatial expression when introduced in excess as competitor. Competition with the whole regulatory domain, while stoichiometrically depressing CAT expression (Livant *et al.* 1988; Franks *et al.* 1990), similarly left the spatial pattern of expression unchanged. An obvious interpretation is that titration onto competitor sequences has sequestered required positive regulatory factors, in-

**Table 2.** DNA fragments of the CyIIIa control region not affecting spatial regulation

Fragment	Total no. scored	Embryos labeled in aboral ectoderm*	No. of embryos with (possible) ectopic expression
P2 (I+II)	59	20	0
P4	48	11	1
P5	113	60	0
P6	70	57	0
P8 (I+II+III)	36	25	1

\* As in Table 1; see note (\*).



**Fig. 1.** Correct lineage-specific expression of the fusion gene *CyIIIa*·*CAT* in pluteus embryos that developed from injected eggs. *In situ* hybridization to 5 µm thick embryo sections was carried out using a single-stranded antisense RNA copy of the *CAT* gene. The probe was labeled with <sup>35</sup>S-UTP in panels a, b, and e–j, and with <sup>3</sup>H-UTP in panels c and d. Embryo sections are shown under phase-contrast and dark-field illumination. (a and b) Adjacent sections of three embryos that expressed *CAT* mRNA in many cells of the aboral ectoderm. The upper and lower right embryos are cut approximately in sagittal section; stomach, intestine and esophagus are visible, and the oral 'face' is toward the right. Analysis of serial sections of the upper right embryo establishes that label is found in cells deriving from 8-cell blastomeres *Na*, *VA*, *NL<sub>R</sub>*, and *VL<sub>R</sub>* (Cameron *et al.* 1987, 1989a). Both the *VA* and *VL* blastomeres also contribute cells to the gut, here completely unlabeled. The lower right embryo appears to be labeled only at the apex, whose cells are clonal descendants of the *VA* blastomere. The remaining embryo (upper left) is cut so that the only tissue seen is aboral ectoderm, except for two small groups of ciliated band cells. (c) Embryo cut lengthwise through stomach and intestine. Oral ectoderm is to the right. The remaining ectoderm, including the only labeled portion, is aboral. The labeling pattern in this embryo indicates that the *Na* blastomere incorporated the exogenous *CyIIIa*·*CAT* gene. (d) Section through aboral ectoderm, intestine and part of the stomach, on the left side of the embryo. This side of the ectoderm (derived from blastomeres *NL<sub>L</sub>* and *VL<sub>L</sub>*) is extensively labeled. *VL<sub>L</sub>* also contributes to the gut, which is not labeled. Both mouth (and oral ectoderm) and anus are several sections away on the slide. (e) This embryo is cut in cross-section through the esophagus and coelomic pouches. A small amount of oral ectoderm is present, adjacent to the esophagus, toward the upper right. The only labeled cells are in the patch of aboral ectoderm on the lower right. (f) Approximately longitudinal cut through the open mouth and esophagus (oral face, left). The aboral ectoderm is almost completely labeled on one side, in cells derived from *NL<sub>R</sub>*. (g) Two embryos cut in cross section. The sections illustrated are toward but not in the oral face, and all the ectoderm seen is aboral. In each embryo one side of the ectoderm is labeled rather extensively, probably in derivatives of an *NL* daughter cell of the 16-cell stage. (h) Two embryos in which only a few cells of the aboral ectoderm are labeled. The embryo on the left is cut in longitudinal section through the intestine. Some *NL<sub>R</sub>* and *NL<sub>L</sub>* (lateral ectoderm) descendants are labeled. The embryo on the right is cut sagittally and includes a tangential section of the stomach. In this embryo also some but not all *NL<sub>R</sub>* and *NL<sub>L</sub>* derivatives are labeled. The oral face of each embryo is toward the upper right of the figure. (i and j) Approximately midsagittal sections of an embryo that had expressed *CyIIIa*·*CAT* in a majority of cells of its aboral ectoderm (as observed in other serial sections not shown). This implies that *Na*, *VA*, *NL<sub>R</sub>*, *VL<sub>R</sub>*, *NL<sub>L</sub>* and *VL<sub>L</sub>* cells all contain the injected gene. Oral face, left. Note that the labeling stops at the ciliated band (thickened cells at the upper and lower edges of the oral face). The sections shown are 5 µm apart. Particular regions of the embryo sections shown are indicated as follows: A, apex; B, ciliated band; C, coelomic pouch; E, esophagus; I, intestine; M, mouth; m, mesenchyme cell; O, oral ectoderm; S, stomach. Oral ectoderm lies between the two lines labeled 'O'; all the ectoderm not labeled as oral is aboral ectoderm.

**Table 3.** Ectopic expression of *CyIIIa*·*CAT* when competing P3 or P7II DNA fragments are injected in molar excess

Fragment (expt)	Total no. embryos*	Total labeled†	Embryos with ectopic label‡	Ectopic as % of labeled embryos
P3 (1)	47	39	20	51
P3 (2)	12	9	2	22
P3 (3)	13	11	6	55
P3 (4)	42	35	8	23
ΣP3	114	94 (82 %)	36	38
P7II (1)	36	35	20	57
P7II (2)	46	43	31	72
P7II (3)	114	110	40	36
ΣP7II	194	188 (97 %)	91	48
<i>CyIIIa</i> · <i>CAT</i> alone§				
(4)	9	9	0	0
(5,6)	137	109 (80 %)	3	3

\* Complete embryos and embryos of which 9 or more serial sections were scored.

† Embryos labeled in two or more sections.

‡ Embryos with ectopic labeling in two or more sections.

§ These results also listed in Table 1, as expts 4, 5 and 6 of that Table. Expts 5, 6 of this series were carried out at the same time and on the same batch of eggs as P3 (4) and P7II (3); and expt 4 similarly served as the internal control for expts P3 (3) and P7II (1).

cluding any that might have promoted ectopic expression had they been present.

#### *Ectopic spatial expression of CyIIIa·CAT caused by competition for P3A and P7II factors*

The key result shown in this paper is illustrated in Figs 2 and 3, and summarized in Table 3. This is that *in vivo* competition with two subfragments of the *CyIIIa* regulatory domain results in a striking ectopic spatial expression of *CyIIIa*·*CAT*. *CAT* mRNA in these samples is detected in lineages that normally never express the *CyIIIa* gene itself, and in which the *CyIIIa*·*CAT* construct remains silent in the absence of competitor (except for 2.5 % or less of possible aberrant cases). One of these subfragments, here referred to as the P3 subfragment, contains two known sites for high specificity DNA-binding proteins, *viz.* the P3A and the P3B sites (Calzone *et al.* 1988; Thézé *et al.* 1990). The P3B site is an octamer protein binding site, and since competitive titration with this site results in embryonic lethality (Franks *et al.* 1990), the endogenous octamer protein is obviously a necessary factor. The site responsible for the specific spatial control function that is perturbed in the P3 competition experiment is most probably the P3A site. Neither a subfragment containing only this site nor the P7II site causes significant competitive decrease in *CyIIIa*·*CAT* expression even when present in what should be sufficient excess (Franks *et al.* 1990), and thus by this test neither functions positively *in vivo*. The *in situ* hybridization results obtained with the P3 and P7II competitions

indicate that in fact both interactions must function *negatively* in normal undisturbed embryos, presumably *preventing* CyIIIa expression in other than the aboral ectoderm cell lineages. When excess molar quantities of either of these binding sites are introduced into the embryos, the result is a failure of these normal repressive interactions, as the factors become sequestered on the competitor fragments. Table 3 shows that from 22% to 55% of embryos in the P3 competition series, and 36% to 72% in the P7II competition series, have at least some cells that display illegitimate expression of CAT mRNA.

The effects of competition with the P3 and P7II subfragments are subtly different. Fig. 2 shows representative examples of ectopic expression with the P3 subfragment. In most cases the ectopic expression is confined to mesenchyme cells, and in some examples a significant fraction of the mesenchyme cells present in the section display CAT mRNA (e.g. Fig. 2d and h). Unfortunately we cannot clearly distinguish skeletogenic mesenchyme from secondary mesenchyme, since the mineral elements of the skeleton are dissolved during preparation. From their positions, however, both would seem to be expressing CAT mRNA: thus for example the active mesenchyme cell along the aboral wall of the embryo shown in Fig. 2h is likely to be skeletogenic, while those applied to the wall of the gut as in Fig. 2c, d, f and g are more probably secondary, but these assignments are certainly not secure. Fig. 2 also includes some very clear examples of labeling in the facial oral ectoderm, which descends entirely from the progeny of the 3rd cleavage No blastomere (e.g. in Fig. 2a, b and e). We did not see any convincing cases among the P3 competition embryos of labeling in the wall of the gut, however. Possible labeling in either stomach or intestine was confined to occasional single cells that could not be verified or supported by labeling in the adjacent sections. Expression occurred in the aboral ectoderm in almost all the P3 competition embryos, whether or not they displayed ectopic CAT mRNA as well, as can be seen, for example in the sections shown in Fig. 2d, e and h. We conclude that the P3 subfragment contains a regulatory element the function of which is required to repress CyIIIa expression in mesenchyme cells and oral ectoderm.

The effects of competition with the P7II fragment, as shown in Fig. 3, are even more dramatic. The central No domain of the oral ectoderm is strongly labeled in Fig. 3a and b (the same embryo seen in adjacent sections), and in Fig. 3c–f; and one side of the ciliary band, which derives from an NL blastomere (Cameron *et al.* 1989a) is strongly labeled in the embryos shown in Fig. 3h and j. Many of the mesenchyme cells to be seen, for example, in the embryo shown in Fig. 3g, are also labeled. Intense labeling of cells in the wall of the gut can be seen in the embryos shown in Fig. 3c, d and i. Again, almost all of the labeled embryos included in the P7II experiments of Table 3 display aboral ectoderm labeling as well, as can be seen in Fig. 3a–h. We conclude that the P7II interaction is required to repress CyIIIa·CAT expression (and thus presumably CyIIIa

expression) in *all* major embryonic lineages except aboral ectoderm. Competitive interference with this interaction produces a catastrophic spatial derangement of expression, in which expression in other lineage element(s) wherein the exogenous DNA resides is added to the normal pattern of aboral ectoderm expression.

## Discussion

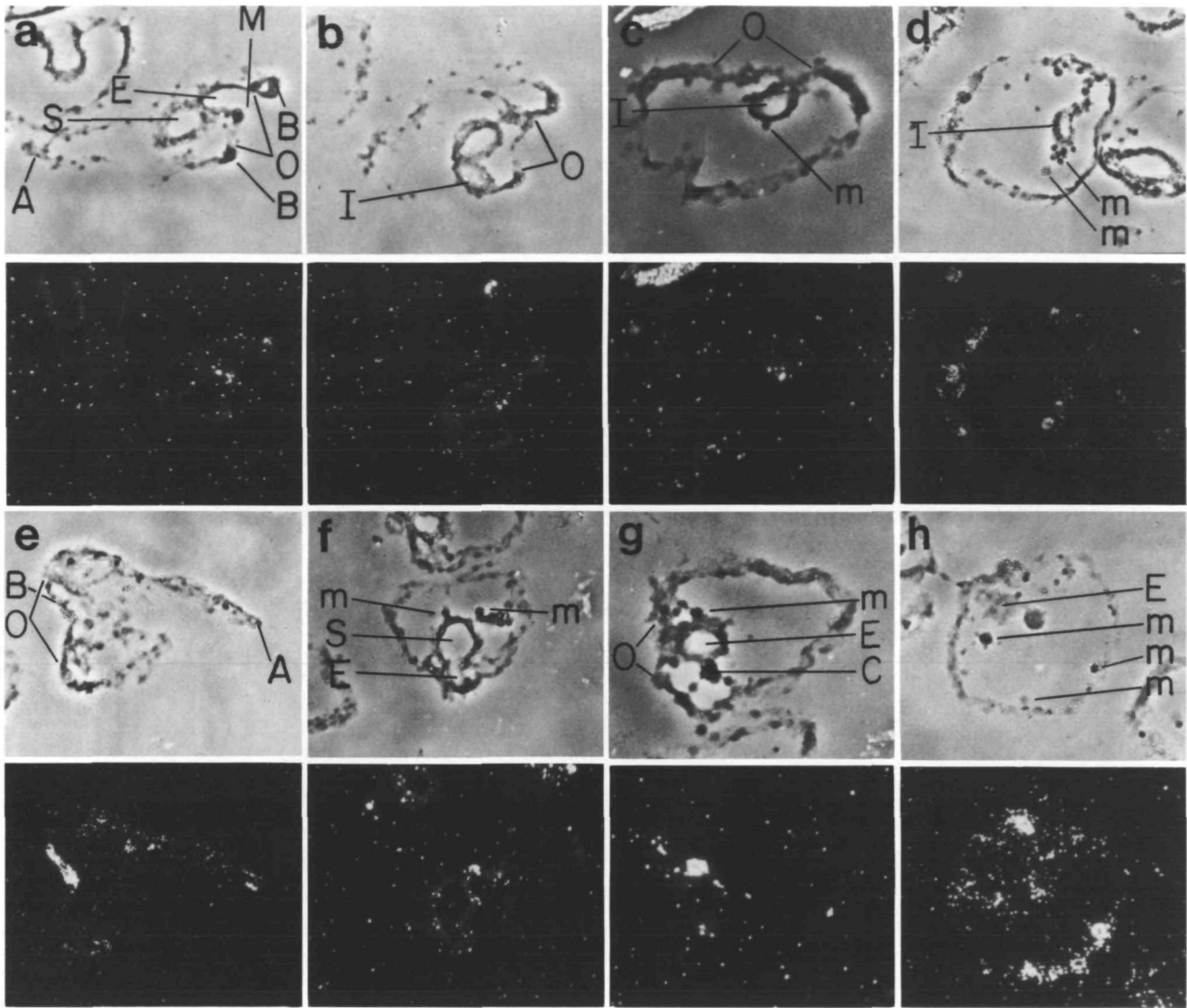
*Negative spatial regulatory functions appear to be confined to the P3A and P7II sites of the CyIIIa gene* Franks *et al.* (1990), in the accompanying study, failed to detect any competitive decrease in expression of CAT mRNA from the CyIIIa·CAT fusion when the P3A or P7II subfragments were coinjected in molar excess. From the quantities of these factors estimated in the embryo nuclei *in vivo* (Calzone *et al.* 1988), it appeared unlikely that this negative result could be due to insufficient excess of the competitor sequence. The results reported here (Table 3 and Figs 2 and 3), demonstrating a frequent, and often extensive ectopic spatial expression of CAT mRNA in the P3 and P7II competition samples, prove that the quantities of stably incorporated competitor sequence indeed sufficed to titrate out the respective factors, at least in some cells of many embryos. It follows that by the competition test these interactions cannot function positively in regulating CyIIIa·CAT expression. Present evidence suggests that the sole function of these interactions is indeed negative, *viz.*, *repression* of CyIIIa expression except in the embryonic territory composed of the eleven aboral ectoderm cell lineages.

We have in this work and that reported by Franks *et al.* (1990) examined subfragments of the CyIIIa regulatory domain that include all of the known sites of high-specificity DNA–protein interaction detected *in vitro* (Calzone *et al.* 1988; Thézé *et al.* 1990). Only one subfragment, that including the two sites bound by the factor P6, failed to result *either* in ectopic spatial expression *or* decrease in CAT expression when coinjected in molar excess with the CyIIIa·CAT fusion. We did not examine the effect of P1 site competition on spatial expression in this work, and it is not excluded that this interaction might also exercise negative spatial control. However, this seems unlikely since, as Franks *et al.* (1990) found, P1 acts as a weak positive regulator of CyIIIa·CAT expression.

### *Spatial regulation of lineage-specific CyIIIa gene expression*

Both of the sites that according to these results would mediate negative spatial regulation of the CyIIIa gene are located close to one or more positively acting sites. This is particularly clear in the case of the P7II site, which is only about 100 nucleotides to the 3' side of the P7I site, that was demonstrated in the accompanying paper to function positively. This is less than the amount of DNA bound by a single nucleosome. Thus as in other systems in which negative regulatory interac-

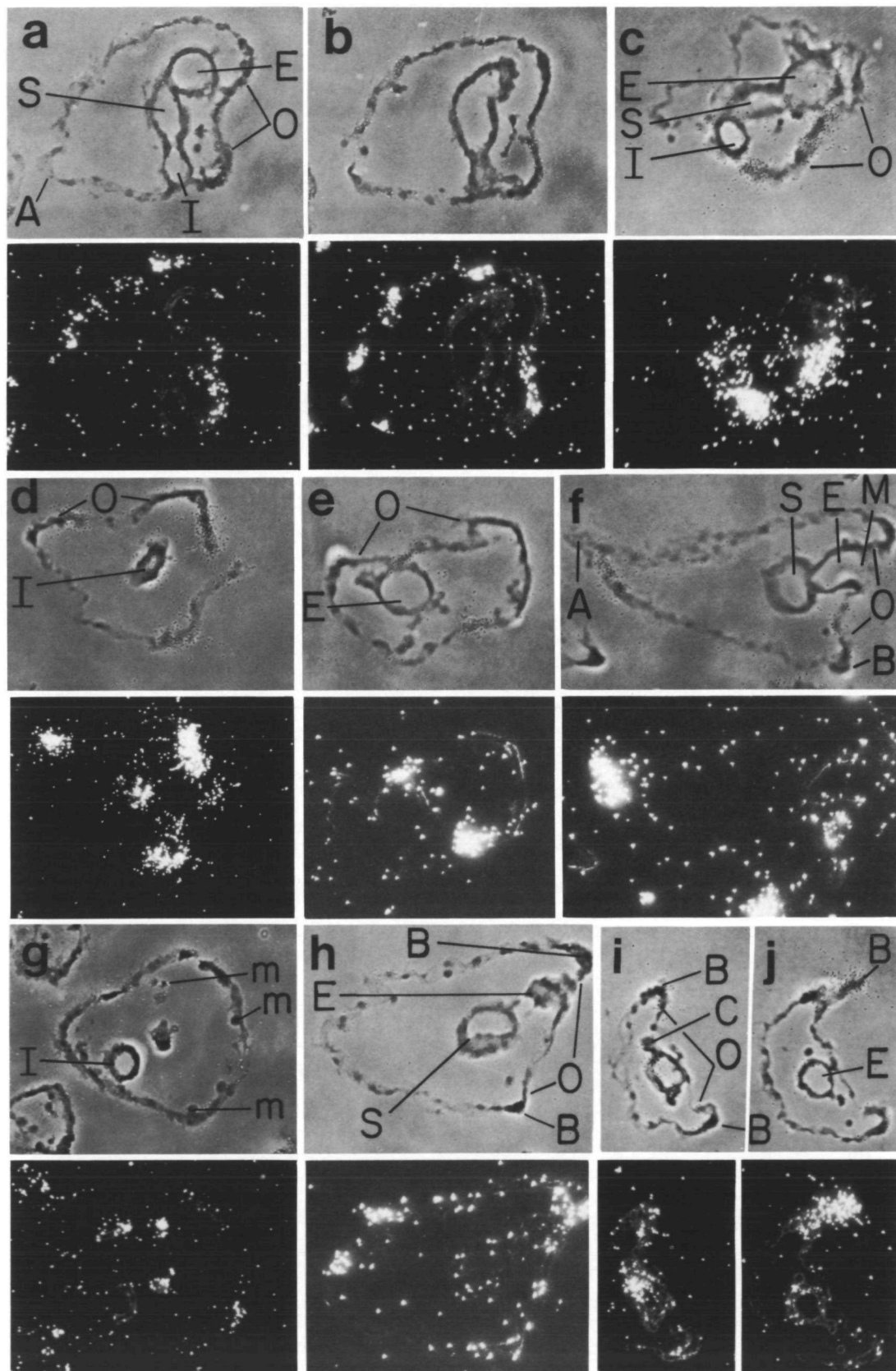




**Fig. 2.** *In situ* hybridization showing ectopic CAT expression in embryos from eggs injected with P3 DNA fragments in competition with the CyIIIa · CAT fusion gene. All the panels except h show hybridization with tritium-labeled CAT probes; in h the probe was labeled with  $^{35}\text{S}$ -UTP. Embryos shown were collected in three completely independent competition injection experiments: (1) a–d; (2) e and h; (3) f and g. Embryo regions shown: A, apex; B, ciliated band; C, coelomic pouch; E, esophagus; I, intestine; M, mouth; m, mesenchyme cell; O, oral ectoderm; S, stomach. (a) Midsagittal section of an embryo labeled in oral ectoderm (near the open mouth) as well as aboral ectoderm (labeling seen in one cell just aboral (left) of the upper ciliated band; in other sections more of the aboral ectoderm was labeled). Esophagus and stomach are visible in this section. (b) Same embryo as shown in panel a, second section further along in the series. The oral ectoderm is labeled in several contiguous sections of this pluteus. (c) Longitudinal section through intestine, oral (upper) ectoderm, and aboral ectoderm. The only labeling is in a mesenchyme cell attached to the gut. (d) Mesenchyme cell labeling in an embryo cut in cross section. The intestine is cut tangentially; all the ectoderm in this section is aboral ectoderm. Two labeled mesenchyme cells are shown. In addition, this embryo is labeled in some cells of the aboral ectoderm. (e) Tangential section through the right side of an embryo in which the ciliary band bordering the oral face is shown on the left, heavily labeled. The remaining tissue in this section is aboral ectoderm. (f) This embryo was sectioned through the stomach and esophagus. A single labeled mesenchyme cell is shown. (g) Longitudinal section (oral face to the left) through esophagus and the wall of one coelomic pouch. A mesenchyme cell above the esophagus is labeled. (h) Embryo similar to that shown in d. Some of the mesenchyme cells seen in the phase photograph are labeled, while others are not. All of the ectoderm in this cross section is aboral. A tangential section of part of the esophagus is completely unlabeled.

tions play an important role, a likely mechanism would be that the negative function operates by interfering with or modifying a physically contiguous positive interaction. The P7I interaction might thus serve as the

essential (positive) element of the repression submechanism mediated by the P7II factor. Similarly, the P3A binding site is but 13 nucleotides distant from the P3B octamer site on the 3' side, and only 90 nucleotides



distant from the potent, positively functioning P5 binding site (Calzone *et al.* 1988; Thézé *et al.* 1990; Franks *et al.* 1990) on the 5' side.

We cannot state on the basis of this or other extant data whether there are positive *as well as* negative lineage-specific controls on spatial expression of these



**Fig. 3.** *In situ* hybridization of sections of pluteus stage embryos that developed from eggs injected with CyIIIa · CAT and P7II competitor. An antisense RNA probe to CAT mRNA was labeled with  $^3\text{H}$ -UTP (panels a, b and g) or  $^{35}\text{S}$ -UTP (c, d, e, f, h and i). Photographs taken under phase-contrast (upper) and dark-field (lower) illumination. Embryos are labeled as in Figs 1 and 2. (a and b) Adjacent  $5\mu\text{m}$  sections of an embryo labeled in both oral and aboral ectoderm. This labeling pattern implies that clonal descendants of **Na** and **No** blastomeres incorporated the CyIIIa · CAT gene. The apex ectoderm and gut are unlabeled; therefore no cells derived from **V** blastomeres contain exogenous DNA. Oral ectoderm is to the right; the three parts of the gut, esophagus, stomach and intestine, are clearly distinguishable. (c) Oblique section through a pluteus in which the intestine and oral ectoderm are labeled, as well as a portion of the aboral ectoderm adjacent to the anus. These regions are derived from the **No** and **VO** blastomeres, indicating that the CyIIIa · CAT DNA was probably incorporated into the oral precursor cell at the 4-cell stage. Additional labeling in the right lateral ectoderm (sections not shown) may mean that some **VL<sub>R</sub>** (right anal plate) descendants also contain CyIIIa · CAT. (d) Gut (intestine) and oral ectoderm labeling in an embryo cut in oblique cross section. The oral face is up; aboral (lateral) ectoderm is also labeled. Our interpretation of the labeling is that some **No** and **NL<sub>L</sub>** daughter cell clones contain the injected gene. (e) Similar section (of a different embryo) cut through the esophagus. The oral face is up. Oral ectoderm (**No**), and aboral (**Na**) ectoderm as well, are labeled. (f) Sagittal section showing aboral ectoderm labeling near the embryo apex. Since the gut is not labeled in this embryo, only apical ectoderm-forming **VA** clones contain CyIIIa · CAT. The open mouth, part of the gut and labeled oral ectoderm (**No**) are to the right. In later sections the right lateral ectoderm (**NL<sub>R</sub>** derivatives) is also labeled. (g) Labeled mesenchyme cells, in an embryo cut in cross section. Aboral ectoderm is also labeled, in a few cells, evidence of a second incorporation event. (h) Pluteus labeled in the upper part of the ciliated band (an oral ectoderm, **No**, derivative) and a few cells of aboral ectoderm. (i and j) Two adjacent longitudinal sections through upper gut (mouth, esophagus and coelomic sac) and arms. Labeling is confined to the right side of the embryo, and is absent from gut and apex; the precursors are descendants of **NL<sub>R</sub>** and **VL<sub>R</sub>**. The oral face is to the right, and the tips of the arms carry cells of the ciliated band. Mouth and the ciliated band of the upper left arm are labeled. In other sections of the same embryo (not shown) the lower left ciliated band, but not the aboral ectoderm, was labeled.

aboral ectoderm-specific genes. Were such to exist, competitive titration *in vivo* (or deletion, or *in vitro* mutations) would merely have decreased or abolished the signal observed, as in the cases recorded in Table 2, without altering the spatial pattern of expression. However, that same result would be expected from competition for factors that have no spatial regulatory functions whatsoever, but serve as enhancers or positive temporal regulators, etc. Even if further experiments reveal that there are indeed lineage-specific, positively acting factors, the negative functions displayed in this study are a dominant regulatory feature, so that loss of these functions ruins the lineage specificity. This is also

seen in the interspecific gene transfer experiment (Franks *et al.* 1988b). The heavily ectopic pattern of CyIIIa · CAT expression observed in *Lytechinus variegatus* embryos in that study strongly resembles that produced in the P7II competition series of Fig. 3 of this paper, in that expression occurs in all major lineage elements. The direct prediction follows that in *L. variegatus* nuclei the P7II factor is either missing or is evolutionarily divergent, so that it can no longer function on the P7II site of the *S. purpuratus* CyIIIa gene. It appears unlikely that failure of the P3A interaction alone to occur would suffice to explain the phenotype of CyIIIa · CAT expression in *L. variegatus* embryos since competitive titration of P3A in *S. purpuratus* does not generate ectopic expression in the gut (Fig. 2). The fact that competitive interference with the P7II interaction alone derepresses CyIIIa · CAT expression in all major lineages suggests that the regulatory system logic is of the form that repression in oral ectoderm and mesenchyme requires *both* the P3A and P7II interactions, while repression in gut requires only the P7II interaction. Alternatively, these two interactions could have similar functions, but there could be such large concentrations of P3A factor in gut cells in particular that the incorporated competitor sequences were quantitatively insufficient to titrate them out.

In summary, the major result of this work is the discovery of lineage-specific, negative regulatory interactions in the sea urchin embryo. These interactions are an essential feature of the mechanism by which is achieved the initial differential spatial expression of a structural gene, that in normal development is transcribed exclusively in the aboral ectoderm lineages of the early embryo.

This research was supported by a grant from the National Institutes of Health [HD-05753]. RWZ is supported by an NIH Training Grant [GM-07616].

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(Accepted 1 June 1990)